

Original Research Article

Crude protein level effects on the sperm quality of Nile tilapia (*Oreochromis niloticus*) broodstock

ABSTRACT

Aims: This work aimed the evaluation of reproductive performance of Nile tilapia (*Oreochromis niloticus*) males, fed diets containing different Crude Protein (CP) levels.

Study design: completely randomized design.

Place and Duration of Study: The present experiment was conducted, in a period of 90 days, at the Hydrobiology and Fish Farming Station of Eletrobrás/ Furnas, in the city of São José da Barra, MG.

Methodology: 15 broodstock males with average age of 30 months were used. They were stocked in ten tanks with dimensions of 8 m³, in which the treatments consisted in five diets with different levels of crude protein (32, 34, 36, 38 and 40%) and with digestible energy per gram of protein of 9.5/kg of feed. For the *in natura* semen experiment, the statistical analysis used completely randomized design. For the experiment of diluted semen, the completely randomized design was also used, with a factorial scheme of 5 x 2, that is, 5 levels of protein and 2 cryoprotectants.

Results: The hepatosomatic indexes (HIS), gonadosomatic indexes (GSI), rate and duration of Nile tilapia males *in natura* semen motility variables were not influenced ($p>0.05$) by the crude protein levels in the diets. After the dilution, significant differences were observed for the motility variables ($p=0.053$) and motility duration ($p=0.021$). Forth and third degree effects occurred for the rate ($p=0.0349$) and duration ($p=0.0220$) motility variables. As these adjustments do not allow a biological interpretation, the Tukey multiple comparison test was used. The diet with 36% crude protein level presented average motility rate similar ($p>0.05$) to the treatments with 32, 34 and 40% crude protein and average motility rate inferior ($p<0.05$) to the treatment with 38% crude protein. For the motility duration variable, the fish fed with 38% and 40% crude protein presented significant difference ($p<0.05$) with only the group of fish fed with 34% crude protein.

Conclusion: It is concluded that 32, 38 and 40% crude protein levels in the diet do not deteriorate semen quality and may be used in tilapia broodstock diet. As protein is an expensive input, it is possible to infer that 32% crude protein is sufficient to determine good quality gametes.

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Keywords: Tilapia; Reproduction; Semen; Cryopreservation; Male

1. INTRODUCTION

Tilapias are the second most important group of fish in world-wide aquaculture. It is highlighted for presenting fast growth rate, rusticity, excellent quality flesh with good market

acceptance and early sexual maturity. While several natural fishing stocks find themselves at their maximum exploration limit, aquaculture's fish production has been increasing in the last few years, in order to attend the market [1-2]. However, the improvement of cultivation techniques along with technological advances in nutrition and feeding is imperative, not only for productive performance, but also in reproductive development of fish, in order to ascertain larger gamete production and larger larvae and fingerling development, which is one of the biggest difficulties for aquaculture development.

Studies in broodstock nutrition are still limited and relatively expensive, due to the necessity of large dimension facilities to maintain large groups of adult fish, and high production costs to conduct prolonged feeding experiments. Among the important nutrients in broodstock diet is the protein, which determines the increase in reproductive performance [3]. However, it is an expensive component in the feed manufacturing process, increasing production costs, making necessary the studies of protein requirement for each species of fish [4-5].

Gonadal development and specially the quality of the gametes, depend on the quality of the diet offered to the broodstock [6]. Proteins are present in fish gametes in the forms of lipoproteins, hormones and enzymes, determining the quality of the egg and large scale fish production [7].

Semen cryopreservation is a biotechnical method used for the maintenance of viable spermatozoa, minimizing problems, such as synchronization between males and females during breeding season, consanguinity and genetic selection [8]. Three factors influence the maintenance of spermatic quality during cryopreservation: freezing and thawing methodology, composition of internal and external cryoprotectants and diluter composition [9].

Thus, this experiment aimed at evaluating the effects of different crude protein levels in the diet of *Oreochromis niloticus* male broodstock, on spermatic quality parameters, as well as evaluating the influence of the cryoprotactants methanol and dimethylsulfoxide (DMSO), in semen freezing.

2. MATERIAL AND METHODS / EXPERIMENTAL DETAILS / METHODOLOGY

2.1 Location

The present experiment was conducted, in a period of 90 days, at the Hydrobiology and Fish Farming Station of Eletrobrás/ Furnas, in the city of São José da Barra, MG, by means of partnership between Universidade Federal de Lavras, Lavras, MG and Eletrobrás-Furnas.

2.2 Animals and diets

Fifteen Nile tilapia (*Oreochromis niloticus*) broodstock males, Chitralada strain, with average age of 30 months were lodged in five masonry tanks with 8 m³ dimensions with continuous water flow and density of three fish per tank. The treatments consisted of feeds of five different crude protein levels (32, 34, 36, 38 and 40%) and digestible energy per gram of protein of 9.5/kg of feed.

Five experimental diets were elaborated (Table 1) with different crude protein (CP) levels, formulated according to a table of chemical composition of the ingredients [10-11]. All the ingredients were ground to the point in which they reached diameter equal or inferior to 0.04

64 mm. The ingredients were weighed, homogenized and added water to, in the proportion of
65 20% of the feed's total weight. The feeds were pelleted in electric feed pelletizer and dried in
66 ventilated oven (50°C), during 24 hours.

67 **Table 1. Ingredients and calculated composition of the experimental diets provided to**
68 **Nile tilápia (*Oreochromis niloticus*) males (based on dry matter)**

Ingredients (%)	Protein levels in the diet (%)				
	ED/g of PB of 9,5				
	32	34	36	38	40
Soybean meal	48.00	53.80	57.50	64.00	68.65
Fish meal (64%CP)	12.00	12.00	12.00	12.00	12.00
Corn	20.90	16.00	15.90	13.04	5.50
Wheat bran	5.00	5.00	5.00	1.00	0.60
Alginate	0.20	0.20	0.20	0.20	0.20
Soybean oil	0.20	2.00	2.00	4.00	7.80
Dicalcium phosphate	4.95	3.99	1.50	0.34	0.45
Vitamin C	0.06	0.06	0.06	0.06	0.06
Salt	0.20	0.20	0.20	0.20	0.20
Vitamin/Mineral Premix ^a	0.50	0.50	0.50	0.50	0.50
Kaulin	3.65	1.61	0.50	0.10	0.10
BHT ^b	0.02	0.02	0.02	0.02	0.02
Total	100.00	100.00	100.00	100.00	100.00
Calculated composition					
Digestible energy (Kcal/kg)	3.040	3.230	3.420	3.610	3.800
Crude protein (%)	32.04	34.24	36.25	38.19	40.05
Ethereal extract	3.87	5.56	5.73	7.64	10.22
Crude fiber (%)	3.74	3.99	4.39	4.30	4.42
Lysine	2.34	2.55	2.54	2.53	2.73
Methionine	0.53	0.55	0.58	0.55	0.58
Threonine	1.44	1.52	1.50	1.46	1.38

69 ^a Vitamin/Mineral Premix: Composition/ kg of the product: vit. A = 900.000 UI; vit. D3 =
70 50.000 UI; vit. E=6.000 mg; vit. K3 = 1200 mg; vit. B1 = 2400 mg; vit. B2 = 2400 mg; vit. B6
71 = 2000 mg; vit.B12 = 4800 mg; folic acid = 1200 mg; calcium pantothenate = 12.000 mg; vit.
72 C = 24.000 mg; biotin = 6,0 mg; colin = 65.000 mg; nicotinic acid = 24.000 mg; Fe = 10.000
73 mg; Cu = 600 mg; Mn = 4000 mg; Zn = 6000 mg; I = 20 mg; Co = 2,0 mg e Se = 25mg. ^b
74 Butylhydroxytoluene (antioxidant).

75 The adaptation periods to the feed and environment were ten days during which the animals
76 received 32% CP feed. The fish were fed twice a day (at 9 and at 15 h), with 2% biomass
77 daily feeding rate. During the experimental period, the broodstock were fed the experimental
78 feeds (Table 1).

79

80 2.3 Limnological parameters

81 The water tanks' limnological variables were measured daily by morning, (conductivity, pH,
82 dissolved oxygen and temperature) by dual channel HQ40D multi-parameter digital probe,
83 according to the Fish Farming Station Eletrobrás/Furnas protocol.

84

85 2.4 Experimental methodology

86 Three males were selected from each tank, being weighed and measured and subsequently
87 transferred to reproduction tanks with 200 L water capacity. For semen extrusion, the
88 broodstock were taken from the tanks, involved in dry towels, and had their urogenital
89 papillae dried with paper towels, to avoid early semen activation through contact with water,
90 feces or urine. The sampling was done through massage in the coelomic cavity in cranium-
91 caudal direction, collected in graduated endendorf tubes.

92 Subsequently were made motility rate (%) and duration (seconds) analysis. The analysis
93 was done in optic microscope, through distilled water activation in dilution of 1:4
94 (semen:water). Motility duration was determined from the time of activation to the time in
95 which 10% of the spermatozoa were mobile, according to methodology used by [12].

96 The semen that presented motility rate above 90% was used for cryopreservation. Two
97 cryoprotectant solutions were used: Solution A: 10% dimethylsulfoxide (DMSO) + 5% BTS
98 (Beltsville Thawing Solution®); Solution B: 10% methanol + 5% BTS. *in natura* semen was
99 diluted in the proportion of 1:4 (semen:cryoprotectant solution). For the toxicity evaluation of
100 the cryoprotectant solutions, were verified spermatoc motility rate and duration, according to
101 methodology used for *in natura* semen, shortly after dilution.

102 The diluted semen was packaged in 0.5 mL pallets and afterward conditioned in cryogenic
103 shipper (Taylor-Wharton Model CP 300, dry shipper type), in which they remained for 24
104 hours, subsequently being transferred to liquid nitrogen where they stayed for a week. After
105 this period the semen was thawed, through immersing the pallets in 60oC water bath for 8
106 seconds. After thawing, the semen was once more evaluated regarding motility rate and
107 duration, according to methodology described for *in natura* semen.

108 After submitted to euthanasia by means of 2-fenoxiethanol solution (0.06%), the males were
109 desiccated and had their liver and gonads weighed and, with this data, the following indexes
110 were calculated:

111 Gonadosomatic index (GSI) = [GSI = (GW+ESW)/FW*100, in which GW is gonad weight,
112 ESW is extruded semen weight and FW is fish weight]; Hepatosomatic index (HIS) = [(liver
113 weight*100)/fish weight].

114

115 2. 5 Statistical analysis

116 For the experiment with *in natura* semen, the statistical analysis proceeded according to a
117 completely randomized design, in which were evaluated five crude protein levels (32, 34, 36,
118 38 and 40%) with three replicates. The evaluated statistical model was:

119

$$120 y_{ik} = \mu + \tau_i + \varepsilon_{ik} \quad (2.1)$$

121

122 in which:

123 y_{ik} is the observation regarding to the i level of the τ factor and to the j level of β factor in
124 replicates k ;

125 μ is the constant associated to all observations;

126 τ_i is the i level effect of the τ factor, with $i=1, 2, 3, 4$ and 5 ;

127 ε_{ik} is the experimental error regarding factor i of replicates k , in which $\varepsilon_{ik} \sim N(0, \sigma^2)$.

128

129 For the experiment with diluted semen a completely randomized design (CRD) was also
130 used in a factorial scheme of 5×2 , in other words, 5 protein levels and 2 cryoprotectants,
131 totalizing 10 treatments with three replicates each according to the following statistical
132 model:

133

$$134 \quad y_{ijk} = \mu + \tau_i + \beta_j + \delta_{ij} + \varepsilon_{ijk} \quad (2.2)$$

135 in which:

136 y_{ijk} is the observation regarding the i level of τ factor and the j level of β factor in the k
137 replicate;

138 μ is a constant associated to all observations;

139 τ_i is the effect of the i level of τ factor, with $i=1, 2, 3, 4$ and 5 ;

140 β_j is the effect of the j level of β factor, with $j=1, 2, 3, 4$ and 5 ;

141 ε_{ijk} is the experimental error regarding all observations, in which $\varepsilon_{ijk} \sim N(0, \sigma^2)$.

142

143 The analysis of variance (ANOVA) for the results was verified by means of the function
144 “shapiro.test” of the “stats” package version 2.15.1 [13] for normality and by means of the
145 function “LeveneTest” of the “car” package version 2.10-12 [14] for homoscedasticity.
146 Having noted differences between the means by means of ANOVA, the sum of squares was
147 unfolded to the forth degree polynomial. The “TukeyC” function of the “TukeyC” package
148 version 1.0-7 [15] was used for the Tukey multiple comparisons test for the variables which
149 polynomial degree was above two. All the analysis were done with the aid of the statistical
150 software R [13].

151

152 3. RESULTS AND DISCUSSION

153

154 3.1 Water quality

155 The average values for temperature (C) of 22.47; conductivity of 36.45; pH of 7.43 and
156 dissolved Oxygen (mg L^{-1}) of 7.02, of the tanks during the experimental period, are found
157 within the limits recommended for tilapia reproduction [16], and are considered adequate for
158 tilapia initial embryonic development [17].

159

160 3.2 Reproductive indexes

161 The reproductive parameters, such as hepatosomatic indexes (HIS), gonadosomatic
162 indexes (GSI), spermatoc motility rate and duration for Nile tilapia males' *in natura* semen,
163 were not influenced $P > .05$ by crude protein levels (Table 2).

164 The HIS may increase depending on the quantity of nutrients in the diet. In this case the fish
165 convert the excess dietary energy to visceral fat [18]. Therefore, the results found for HIS in
166 this study suggest the protein in the diet did not influence this variable. Similar results were
167 reported by [19] that, working with 28, 34 and 40% crude protein levels in diets for *Rhamdia*
168 *quelen* female broodstock, did not find statistical difference for liver weight.

169
170 **Table 2. Values mean ($\pm$ standard deviation) of Gonadosomatic indexes (GSI),**
171 **hepatosomatic indexes (HIS), spermatic motility rate (MOT) and spermatic motility**
172 **duration (DUR) of *in natura* semen of tilapia broodstock submitted to feeding with**
173 **different crude protein levels**

Treatment (%)	GSI	HIS	MOT%	DUR (s)
32	0.70 $\pm$ 0.37	1.43 $\pm$ 0.34	98.33 $\pm$ 2.89	450.00 $\pm$ 132.07
34	0.42 $\pm$ 0.73	0.64 $\pm$ 1.10	96.67 $\pm$ 5.77	1051.67 $\pm$ 574.65
36	0.93 $\pm$ 0.07	1.54 $\pm$ 0.18	98.33 $\pm$ 2.89	780.67 $\pm$ 370.30
38	0.54 $\pm$ 0.55	0.98 $\pm$ 0.85	100.00 $\pm$ 0.00	1078.67 $\pm$ 241.87
40	0.87 $\pm$ 0.90	0.91 $\pm$ 0.80	98.33 $\pm$ 2.89	726.00 $\pm$ 344.71

174
175 The GSI was used to monitor gametogenesis progression in teleost fish [20]. In this study
176 the GSI was not affected by the increase in diet crude protein levels (32, 34, 36, 38 and
177 40%). These results are similar to those reported by [21], when protein levels of 10, 20 and
178 35% were evaluated for tilapia.

179 After the semen was diluted, there was no significant interaction ($P > .05$) between crude
180 protein levels and cryoprotectants for any of the evaluated variables. There was significant
181 difference only between crude protein levels for motility ($P = .05$) and motility duration ($P =$
182 $.02$) variables. A significant ($P < .05$) forth degree polynomial adjustment was observed in
183 crude protein levels for the motility variable and a significant ($P < .05$) third degree
184 polynomial adjustment on crude protein levels for the motility duration variable. The Tukey
185 test was done since this adjustment does not refer to a biological interpretation. For motility,
186 32, 34, 38 and 40% protein levels were similar ($P > .05$), however, the fish fed 38% crude
187 protein presented larger motility than the fish fed 36%. For the motility duration variable, the
188 fish fed 38% crude protein presented significant difference ($P < .05$) only to the group of fish
189 fed 34% crude protein (Table 3).

190
191 **Table 3. Motility means (%) and motility duration (s) after the dilution of the semen**
192 **from tilapias fed different protein levels and with different cryoprotectants**

Protein levels	Motility			Motility Duration		
	Cryoprotectants					
	DMSO	Methanol	General Mean*	DMSO	Methanol	General Mean*

32	96.7±5.8	90.0±10.0	93.3±8.2 ab	811.7±295.4	514.7±289.0	663.1±125.6 ab
34	97.5±3.5	81.7±12.6	88.0±12.5 ab	367.0±86.3	256.3±72.7	300.6±40.4 b
36	78.3±24.7	73.3±37.9	75.8±28.7 b	807.0±518.8	463.3±453.6	635.1±193.8 ab
38	100.0±0.0	100.0±0.0	100.0±0.0 a	1103.7±125.4	956.7±163.6	1030.1±62.5 a
40	95.0±5.0	88.3±7.6	91.7±6.8 ab	927.7±462.6	971.3±503.9	949.5±176.9 a
General Mean	93.2±13.1	86.7±18.3	89.8±16.1	834.6±378.7	632.5±410.8	730±401.8

* Means followed by distinct letters in the column, differ significantly by Tukey test ($P < .05$).

194

195 Some papers related to tilapia broodstock nutrition demonstrated that spermatic quality is
 196 influenced by the diet supplied to the broodstock [22]. In the present study, the results
 197 demonstrated that crude protein levels in the feed did not alter motility parameters and
 198 motility duration of fresh semen. The semen's quality is measured by seminal composition
 199 and motility [23], this is because after spermatic activation the spermatozoa must be capable
 200 of reaching the oocyte surface and penetrate the micropyle [9].

201 As such, the better the seminal quality, the larger the probability to obtain a high fertilization
 202 rate, given that the fresh semen in all the treatments reached average motility above 96%.
 203 Through the cryoprotectant solution toxicity test, subsequent to semen dilution, it was
 204 possible to observe that the motility rates remained above 70%. The spermatic motility
 205 evaluation after freezing is necessary to verify the cryoprotectant's efficiency in maintaining
 206 seminal viability post-freezing. The cryopreservation process may cause injuries to the
 207 spermatic cells, which results in decreasing of spermatic quality [12]. When the crude protein
 208 levels of the cryoprotectants are compared, the results of the present study are similar to
 209 those reported by [24], that did not find significant difference working with methanol or
 210 DMSO for tilapia semen. Motility and motility duration behavior of the semen after freezing
 211 with different cryoprotectants may vary depending on the species. Quote that DMSO is the
 212 cryoprotectant most used in the majority of the cryopreservation protocols for semen in
 213 concentrations that range from 5 to 15% [25]. Meanwhile, methanol has proved more
 214 effective for species such as *P. corruscans* [26].

215

216 4. CONCLUSION

217

218 With these results it is possible to infer that the crude protein levels in the diet influenced the
 219 spermatic quality patterns enough to determine an effective semen cryopreservation. Given
 220 that 32, 38 and 40% crude protein levels in the feed did not deteriorate semen quality and
 221 may be used in the feed for tilapia broodstock. Because protein is an expensive input,
 222 broodstock may be fed 32% crude protein, without loss in spermatic quality.

223 However, future studies must be done focused on broodstock nutrition, to elucidate the
 224 effects crude protein have on semen cryopreservation, to determine this nutrient's efficiency
 225 after semen freezing, attempting to increase seminal cryoresistance.

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